Independent regulation of anterior/posterior and equatorial/polar polarity in the *Drosophila* eye; evidence for the involvement of Wnt signaling in the equatorial/polar axis

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SUMMARY

The *Drosophila* retina is made from hundreds of asymmetric subunit ommatidia arranged in a crystalline-like array with each unit shaped and oriented in a precise way. One explanation for the precise cellular arrangements and orientations of the ommatidia is that they respond to two axes of polarized information present in the plane of the retinal epithelium. Earlier work showed that one of these axes lies in the anterior/posterior (A/P) direction and that the polarizing influence is closely associated with the sweep of the Hedgehog-dependent morphogenetic wave. Here we present evidence for a second and orthogonal axis of polarity, and show that it can be functionally separated from the A/P axis. Further, we show that the polarizing information acting in this equatorial/polar axis (Eq/Pl) is established in at least two steps — the activity of one signaling molecule functions to establish the graded activity of a second signal.

Key words: *Drosophila*, Retina, Ommatidia, Polarity, Axis, Signaling

INTRODUCTION

Planar polarity (Nübler-Jung, 1987) is a characteristic displayed by epithelia in which the cells are all coordinately aligned so, for example, they may project their hairs or bristles in the same direction. In such a situation, cells separated by many hundred cell diameters are able to coordinate their behaviour, raising the question of how such long-range organization is established during development. It may be that distant cells are able to communicate with each other and so coordinate their behaviour or, alternatively, cells may organize their behavior independently of each other by responding to a distant polarizing signal. The phenomenon of planar polarity therefore provides an attractive system with which to study the mechanisms of long-range developmental organization.

Planar polarity has been extensively investigated in the insect (largely hemiptera) ectoderm through surgical manipulation techniques (e.g. Piepho, 1955; Lawrence, 1966; Stumpf, 1967; Nübler-Jung, 1987) and recently by molecular and genetic manipulations in *Drosophila* (e.g. Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987; Struhl et al., 1997). The eye of *Drosophila* differs from many other epithelia in that planar polarity is not evident by the orientation of single cells but rather by the shape and orientation of the ommatidia, which are small clusters of cells.

Ommatidia are small groups of cells arrayed precisely and interlocked to form a retina of crystalline-like structure. The ommatidia contain a number of asymmetries, but the most obvious is the shape assumed by the rhabdomeres of the photoreceptors. The rhabdomeres in cross-section appear as large blob-like structures (Fig. 1A-C), and the so-called outer rhabdomeres (those corresponding to photoreceptors R1-R6) form an asymmetric trapezoidal shape when viewed in cross-section. The trapezoids occur as one of two different chiral shapes (color coded here as red and blue for simplicity, Fig. 1A-C). Those in the dorsal hemisphere are all of one type and are the mirror reflection (the other chiral form) of those in the ventral hemisphere (Dietrich, 1909), (Fig. 1C). The two forms meet at the line of pattern inversion termed the equator and in keeping with this global metaphor the dorsal and ventral extremities of the eye are called the poles. In the right eye the red form is found dorsally, but in the left eye the blue form is found dorsally, reflecting the mirror symmetries of the left and right eyes (Fig. 1D). Thus, the chiral shapes of the ommatidia do not correlate with a dorsal or ventral location; rather, they respect other positional information present within the eye.

Epithelia are largely cellular monolayers and we envisage them as two-dimensional sheets. Conceptually, the uniform orientation of cells in a two-dimensional sheet requires only a single organizing signal — the wind to align all the weather vanes or the magnetic field that uniformly orders the monopoles. But the ommatidia are asymmetric structures within the sheet and so require two pieces of shape-organizing information. A number of models can be proposed to explain how ommatidial fields containing the same chiral (color) shape can be achieved but the simplest is that there are two orthogonal planar polarities in the developing retina (Wehrli and Tomlinson, 1995). In this model, one planar polarity runs in the A/P axis of the retina and the other...
runs between the equator and the pole (Eq/Pl) (Fig. 1D-2,3). The combined directional signals of the two polarities then direct each ommatidium in one eye half into the same shape (Fig. 1D-4). The mirror reflection of the Eq/Pl signal about the equator directs the ommatidia of the dorsal and ventral eyes into the opposite shape, and the mirror reflection of the A/P signal between the right and left eyes inverts the shapes between one eye half and its corresponding hemisphere in the other eye (Fig. 1D-4).

Evidence for the two-planar polarity model (we call this cruciform planar polarity) came from experiments in which the morphogenetic wave that normally traverses the retinal epithelium from posterior to anterior in late larval development was reversed. When the wave was induced to run backwards (from anterior to posterior), then a concomitant reversal of the A/P axis of the ommatidia was observed. Here the ommatidia were still oriented correctly in the Eq/Pl axis, but were reversed in the A/P axis (Heberlein et al., 1995; Ma and Moses, 1995; Strutt et al., 1995; Wehrli and Tomlinson, 1995). Thus, since A/P information could be reversed without interfering with Eq/Pl polarity, then this argued in favor of the separate polarities. However, other results (Chanut and Heberlein, 1995; Strutt and Mlodzik, 1995) argued that the moving wave front, in addition to organizing the A/P polarity, could also provide the Eq/Pl information. This suggested that the signals polarizing the Eq/Pl axis were intimately associated with A/P-organizing mechanism, arguing against the idea that two separate polarizing mechanisms are superimposed to direct ommatidial chirality. To resolve this issue, we designed experiments to test whether Eq/Pl information can be manipulated without affecting A/P polarity.

We show here that Eq/Pl polarity can be manipulated without disturbing the A/P axis which we offer as further evidence for the two separate polarizing mechanisms. The wave front that lays down the A/P polarity is propagated by a mechanism that utilizes both the Hedgehog and TGF-β-type of secreted factors. We therefore looked at other sequestered molecules and present evidence here that Eq/Pl polarity appeared to be controlled by a Wnt signaling mechanism. The Eq/Pl-organizing mechanism appears to be controlled primarily by Wg (or another Wnt) signaling that controls the expression of a secondary (unidentified) signaling molecule. We propose that the graded activity of this secondary molecule in the Eq/Pl axis of the retina provides the positional information for polarizing the ommatidia in this axis.

**MATERIALS AND METHODS**

**Histology**

Eyes were processed for sectioning and analysis following Tomlinson and Ready (1987).

** Constructs and misexpression**

**white** flip-out cassettes

The initial wg experiments used a flip-out cassette containing the mini-white gene (Klementz et al., 1987); this construct was a gift of Gary Struhl. Since this frequently gave only low level pigmentation in the eyes, we constructed a version expressing the white gene more strongly. Here the white cDNA (Pepling and Mount, 1990) was subcloned from the 5′ end of the coding sequence to the ApaI site (immediately upstream of the translational stop) and joined to the 3′ end of the mini white gene from the ApaI site to the end. The GMR enhancer element with the hsp70 minimal promoter (Hay et al., 1994) was placed immediately 5′ of the coding sequence and the whole construct was flanked by two FRT sequences.

**wg and Wnts**

The wg coding sequence carrying the tubulin trailer was subcloned into a transformation vector containing the Tubulin-tol promoter (Basler and Struhl, 1994) and the mini-white flip-out cassette was inserted between the wg coding sequence and the tubulin promoter. Similar constructs were made for Wnt-2, Wnt-3 and Wnt-4 cDNAs (Eisenberg et al., 1992; Russell et al., 1992; Graba et al., 1995). Ectopic expression of the proteins was induced by crossing the transformed lines to flies carrying flipase under the transcriptional control of the hsp70 promoter and these were heat-shocked at various times.

**Activated Armadillo**

A truncated arm coding sequence (missing the codons for the N-terminal 20% of the protein (Zecca et al., 1996) was subcloned into the tubulin vector described above. Two derivative constructs were made by removing the tubulin promoter and inserting either UAS or the sevenless enhancer (two sev enhancers followed by the hsp70 promoter; Basler et al., 1991). Into each of the three constructs, the white* flip-out cassette was inserted between the promoter element and the coding sequence. The UAS construct was driven by GAL4 under the transcriptional control of the arm promoter (Sanson et al., 1996).

**Mosaic analysis**

**arrow**

All data shown were produced with the arr2 allele and the repolarizing effects have been reproduced with arrG6 and arrG68. Clones were induced following Wehrli and Tomlinson (1995) in flies of the genotype y, w; hsp-flipase; FRT42D arr, bw, sp/FRT42D w*(47A). arr2 was obtained from the Bloomington Stock Center and arrG6 and arrG68 were a gift of J. Jiang and G. Struhl (unpublished).

**arm**

armEM19 (Wieschaus et al., 1984), arm25B (Riggleman et al., 1989) and armH64 were all examined in clones in the eye. Partially rescued arm clones were generated in the following flies:

- y, armEM19, w; BCD7, FRT42D, bw/FRT42D P[arm]+/E, P[w160C]FL122+/+
- BCD7 is an arm rescue construct inserted at 36A that only partially rescues the arm phenotype (Peifer et al., 1991). P[arm]+/E is an arm genomic rescuing construct (gift of M. Peifer). FL122 is a hsp70-flipase on the third chromosome (gift of Gary Struhl).

**shaggy**

X-ray clones were induced in females of the following genotype – w1118, sggs=M11+/+, sggs=M11 was described in Perrimon and Smouse (1989). sggs- wg− clones were induced in flies of the following genotype: sggs=M11 w; Dp(1;2)sca19 y*, Dp(1;2) w70h (sggs w*), FRT39E/w8CX4 FRT39E; FL122+/+

**dishevelled**

Clones were induced in females of the following genotype – w1118, dsh129/FRT18A; FL122+/+, dsh129 was described in Klingensmith et al. (1994). We further determined that the open reading frame between nucleotides 496 and 1040 is deleted. The nucleotide sequence across this junction is tgtgaccatAacgccc. A single thymidine is inserted in place of the deleted region. This results in a frame shift after amino acid 94 of Dsh, thus the product lacks the C-terminal 529 amino acids. C-terminal to the truncated Dsh protein, the shifted frame adds 111 amino acids of non-Dsh protein sequence.

**RESULTS**

**Misexpression of Wg reverses Eq/Pl polarity**

The A/P polarity in the retina is laid down with the sweep of the morphogenetic wave that is driven by the TGFβ and Hedgehog
families of secreted factors (Heberlein et al., 1993; Ma et al., 1993). Assuming that secreted factors would also be involved in Eq/Pi polarity, we therefore looked for a role of the another major class of secreted proteins, the Wnts, in organizing the Eq/Pi axis. Wg was ectopically expressed in clonal patches under the transcripational control of the Actin5C promoter (Struhl and Basler, 1993) and significant changes in retinal polarity were observed (Treisman and Rubin, 1995; Tomlinson et al., 1997). The flip-out cassette used in these experiments was yellow*, which is not a useful marker in the retina and cells ectopically expressing it could not be detected. Also, it appeared that cells ectopically expressing Wg did not differentiate and only a scar was present to mark their position in the adult retina. We were concerned that the polarity effects observed may have been related to a secondary effect of the scarring rather than as a direct result of the Wg misexpression. We changed the construct of Struhl and Basler (1993) by substituting the Tubulin-α1 promoter (for the Actin5C promoter) and replaced the yellow+ flip-out cassette with a (mini)white+ flip-out cassette.

Misexpression of Wg with the Tubulin-α1 promoter
We ectopically expressed Wg in the developing eye using the Tubulin-α promoter and marked the clones by the loss of the pigment-conferring white gene. Under these conditions, we observed two significant differences to the earlier experiment described above. First, large white patches of healthy ommatidia were generated with associated polarity inversions and no scarring occurred. This demonstrated that the polarity inversions resulted from the Wg misexpression rather than some secondary defect related to the scarring of the tissue. Second, although significant changes in retinal polarity were associated with the clones, the distance over which the effect was exerted was diminished from a maximum of 6 or 7 ommatidial rows in the Act5C-wg scars down to a maximum of about 1 or 2 for the Tub-wg patches. Small clones rarely showed polarity phenotypes, only those containing about 20 ommatidia or above had associated effects on chirality.

Ectopic Wg clones had two distinct features in respect to their polarity effects. First, the aberrant polarity was asymmetrically distributed with relation to the clone. Changes in polarity occurred at a polar position relative to the center of the clone extending into the wild-type tissue lying outside the clone on the polar side. The ommatidia in the equatorial region of the clone and in the bordering wild-type tissue remained unaffected. Fig. 2B shows the effect of a Wg-expressing clone and the asymmetrical distribution of the polarity reversals is evident as the ‘color change’ of the ommatidia. Note that the ommatidia are inverted in the Eq/Pi axis (rather than A/P) as they still maintain their A/P orientation but now point down to the equator rather than up to the pole. Second, the potency of the Wg-expressing clones to induce polarity reversals showed a change along the extent of the Eq/Pi axis and suggested that the retina has a graded sensitivity to misexpression of Wg, with maximal polarity-reversal effects at the equator and minimal effects at the pole. We scored 30 clones containing 20 ommatidia or above and we classified these into four different types depending upon their position in the eye. We observed 3 clones that spanned the midline of the eye and these caused a major reorganization in the equatorial region. They induced polarity reversions leading to two equators separated by a medial pseudo-equator (Fig. 2C; see Fig. 8C for an explanatory diagram). 18 clones lay between the equator and the pole (Fig. 2B, see Fig. 8B for an explanatory diagram) and, of this class, those closer to the equator tended to induce greater polarity inversions than those closer to the pole. 6 clones were restricted to the polar region and showed no polarity inversions (Fig. 2C). The final class of clones were large clones that extended from the pole to more than half-way to the equator. Whether Wg normally functions to organize the Eq/Pi axis or whether it mimics another activity is discussed later.

The roles of the elements of the Wg transduction mechanism in Eq/Pi polarity
Since misexpression of Wg produced significant polarity effects, we then examined the roles of proteins associated with the Wg transduction pathway. A number of proteins have been implicated in Wg signaling. These included Dishevelled (Dsh), Shaggy (Sgg, otherwise known as Zw3) and Armadillo (Arm). More recently the product of the arrow (arr) gene has been
placed in the Wg signaling pathway on a number of criteria. First, mutant embryos display a segment polarity phenotype (Nüsslein-Volhard et al., 1984). Second, not only does the segment polarity phenotype appear like Wg, but in all other tissues examined (gut, leg, wing) arr phenotypes phenocopy eg mutants (S. Dougan, L. O’Keefe, K. Caldwell and S. DiNardo, pers comm.). Third, an epistasis experiment performed in the eye places arr downstream of Wg. To demonstrate this, we misexpressed Wg in the developing eye using the GMR enhancer element (Hey et al., 1994). The GMR enhancer drives at high levels in cells posterior to the morphogenetic furrow and this induced a very small eye consisting almost entirely of pigment cells. We then induced arr mutant clones in these eyes and observed an autonomous rescue of the ommatidia (Fig. 3).

To examine whether the Wg pathway normally regulates the Eq/Pl polarity in the eye, we manipulated all four of these Wg transduction elements (Dsh, Sgg, Arm and arr).

**Analysis of arrow**

In the eye, arr mutant clones had two distinct effects that occurred at roughly equal frequency. First, arr clones induced ectopic differentiation of the retina ahead of the morphogenetic furrow, in a similar manner to ptc clones or ectopic expression of Hedgehog (see Wehrli and Tomlinson, 1995; Heberlein et al., 1995; Strutt et al., 1995). Ectopically differentiating patches of retinal tissue cause a radial wave of morphogenesis to propagate in the epithelium with concomitant A/P repolarization. Thus any Eq/Pl repolarization that occurred with these clones was obscured by the effects of the wave. The reason why arr clones were able to induce ectopic differentiation is discussed later and, since analysis of these clones does not aid in the investigation of the Eq/Pl axis, we will not describe them further. Second, arr mutant clones that did not induce ectopic differentiation showed clear repolarizing effects in the Eq/Pl axis and this is the class that we describe below.

**arrow clones cause non-autonomous polarity inversion in the Eq/Pl axis**

In the eye, arr clones had three significant features with regard to their influence on the Eq/Pl axis and these occurred in both dorsal and ventral halves of the eye. First, they had clear non-autonomous effects that caused the inversion of Eq/Pl polarity in neighboring and distant wild-type ommatidia. Second, these polarity inversions were only found on the equatorial side of the patch and the polar side remained unaffected. The polarity inversion on the equatorial side of the clone was the opposite to that seen with ectopic Wg which induced the inversions on
do not display repolarizing activity. (C) Clones at the equator described for A, an ectopic equator forms clone are of the correct chiral shape and as distance. Note that ommatidia polar to the in the Eq direction but over a shorter intermediate Eq/Pl position still repolarizes equator. (B) A clone positioned in an pointing up, thereby creating an ectopic clone the ommatidia organize with the blue form pointing down and the red type clone the ommatidia organize with the blue form pointing down and the red type.

The enhancer trap Eq-1 (Sun et al., 1995) displays differential clone the ommatidia organize with the blue form pointing down and the red type.

the polar side. Third, the extent of polarity inversions induced by arr clones was position-dependent. At the poles, the clones exerted their maximal influence into the wild-type tissue, inverting the polarity over many ommatidial rows. This effect diminished progressively with the distance of the clone from the pole, disappearing at the equator (Fig. 4A-C).

Within the clone, the chiralities of the ommatidia were not randomly arranged. In the equatorial region of a clone, the ommatidia are inverted into the inappropriate chiral form found in the neighbouring wild-type tissue. But, in the polar regions of the clone, the ommatidia are of the correct chiral form (the form found polar to the clone) and thus within these patches of arr tissue, ectopic equators are present (Fig. 4A-C).

arr clones activate an equatorial marker

The enhancer trap Eq-1 (Sun et al., 1995) displays differential white+ activity in the Eq/Pl axis – pigment is expressed at high levels at the equator and this grades off rapidly towards the poles (Fig. 5A). Thus the presence of the pigment marks tissue at the equator. In eyes carrying (unmarked) arr mutant clones, we saw ectopic expression of the equatorial marker (Fig. 5B). To demonstrate that the ectopic expression of the marker correlates with arr mutant clones, we sectioned these eyes and observed the chirality changes characteristic of an arr clone (data not shown). As a complementary experiment, we induced clones of arr marked with white in flies carrying the Eq-1 enhancer. When we sectioned such clones that were distant from the equator, we saw the ectopic expression of the equatorial enhancer evident as a low level of pigment within the cells of the clone (Fig. 5D). Hence this equatorial marker becomes ectopically expressed in arr clones.

The non-autonomy of the arr clones is significant since it argues that arr functions to regulate the activity of a downstream signaling molecule that we term factor-X. From this we propose two distinct elements in the Eq/Pl-organizing mechanism. First, there is the step that organizes the polarity signals and, second, there is the process by which the ommatidia ‘read’ those signals. If the signals cannot be read by the ommatidia then we predict that chiral shapes within a clone will be randomized. Conversely if a gene product is used in the polarity-organizing step then mutant clones will show non-autonomous effects outside the clone. If a gene product is used in both steps, then ommatidia within a clone would be randomized and non-autonomous polarity inversions would be evident in wild-type tissue outside the clone. From this perspective, the non-autonomy of arr clones placed this gene in the polarity-organizing mechanism, but the orderly array of the ommatidia within the clones suggests it was not involved in the read-out step. Since arr appears to transduce the Wg signal then our results suggested that Wg signaling through arr may function to regulate the activity of the subservient signaling molecule, factor-X (see Fig. 8).

We then asked whether experimental manipulation of other Wg transduction molecules would confirm this view.

Analysis of Armadillo

In the conventional view of Wg signaling, the loss of Arm prevents the transduction of the signal, whereas activation of Arm can mimic the receipt of the Wg signal. If Arm, like arr mediates the Wg regulation of factor-X activity, then we could make two predictions. First, loss of Arm should mimic arr and result in a repolarization on the equatorial side of a mutant patch. Second, since activation of Arm occurs in response to Wg signaling then, if we expressed an activated form of Arm, it should behave like misexpression of Wg and cause polarity inversions on the polar side of the mutant patch.

Reduction of arm function changes polarity on the equatorial side

We induced arm clones marked with white in the eye using a strong allele (armXM19) and also two weaker alleles (arm25B and armH6-1425). Only extremely small and infrequent clones were
ever observed. They were more frequent with the weaker alleles and tended to be in the equatorial rather than the polar regions of the eye and, when sectioned, white photoreceptors were not observed. Sometimes however, short-range Eq/Pl polarity inversions were associated with these clones.

To generate viable clones, we made use of BCD7, a genomic arm rescue construct (Peifer et al., 1991) that expresses only weakly due to a position effect. We induced arm XM19 clones in the presence of the BCD7 insertion and observed large clones with associated Eq/Pl polarity inversions. We regarded these as clones of a pseudo-hypomorphic arm allele. Some clones showed evidence of the ectopic differentiation of the retinal tissue that we had observed also with arr clones and the significance of this will be addressed in the Discussion. More importantly, in terms of the theme of this paper, the arm clones frequently showed the polarity inversions on their equatorial side. Fig. 6A shows such a clone and the polarity reversal is evident as the ectopic array of blue ommatidia in the lower half (Eq) of the clone. The polarity inversions were usually contained within the mutant patch, but when the clones were induced in dorsal regions clear non-autonomy could be detected and wild-type ommatidia up to two or four ommatidial rows distant from the clone showed chirality changes. We were unable to assay the expression of the equatorial marker Eq-1 in these clones since the BCD7 partial rescue construct is marked with white+ and this would obscure any ectopic expression of the marker. From these results, we inferred that Arm, like arr, is involved in the transduction of the signal that leads to the regulation of the secondary signal (factor-X).

To test further the proposal that Arm function regulates factor-X activity, we now expressed an activated form of Arm, which we predicted would mimic Wg and lead to polarity inversion on the polar side of the clone.
Clones of activated Arm change polarity on the polar side

To examine the effects of activating Arm rather than reducing its function, we misexpressed a N-terminal truncated version of the protein known to elicit activation of the Wg signaling pathway (Zecce et al., 1996). We engineered a UAS construct that allowed the expression of this ‘activated’ form of Arm using the GAL4 system (Brand and Perrimon, 1993) when an intervening white* flip-out cassette was removed. We then used flipase to induce white clones that expressed the activated Arm under the transcriptional control of a ubiquitous GAL4 line (AG11 – Sanson et al., 1996) In the center of these clones no photoreceptors formed but towards their periphery mutant ommatidia containing reduced numbers of photoreceptors were evident. To score the chirality of an ommatidium requires all eight photoreceptors to be present and so the polarity of the tissue in these regions could not be assessed. But, at the interface with the wild-type tissue, normal ommatidia formed and here we observed that polarity inversions only occurred at the polar interface with the wild type tissue and not in the corresponding equatorial position. Also these polarity inversion effects only occurred in clones induced in the equatorial regions and not in the more polar positions. Thus activated Arm clones behaved like ectopic expression of Wg but in a weaker manner – they induced polarity defects on the polar side and clones were more potent to induce polarity inversions in the equatorial rather than polar regions. Also the failure to form photoreceptors within the center of the clones appears similar to the effect seen with misexpression of Wg with the Actin5C promoter, which causes the scarring of the tissue.

These results suggested that both arr and Arm were involved in regulating the activity of the secondary signal – factor-X. If Sgg also was involved in this process, and since cells lacking sgg normally behave as though they had received the Wg signal, then we predicted that sgg clones in the eye would appear like both ectopic Wg expression and activated Arm in that they would cause polarity inversions on the polar side of the clone.

Analysis of Shaggy

Shaggy clones induce polarity reversals on the polar side of the clone

When sggM11 clones were induced in the eye, scars occurred similar to those seen when Wg was misexpressed with the actin5C promoter and no white photoreceptors were present within the scarred tissue. However, outside the scarred area, ommatidial chirality was inverted on the expected polar side of the clone (Fig. 7C). Since the sgg clones phenocopied the effects of ectopic Wg, we were concerned that sgg clones might cause polarity effects by ectopically transcribing wg. We therefore induced sgg and wg double mutant clones and observed that the phenotype remained unchanged (data not shown). Thus ectopic Wg-expressing clones, patches of activated Arm and clones of loss of sgg function all behave in a similar manner and induce repolarization on their polar side. This result is in marked contrast to the arr and arm reduced-function clones which repolarized on their equatorial side.

Separating the polarizing mechanism from the polarity read-out

The results presented so far have related to the mechanism that organizes the polarizing influence within the Eq/Pl axis of the retina. Mutations such as frizzled (fz) and dishevelled (dsh) appear to randomize the chirality of the ommatidia. That is, the developing ommatidia appear unable to determine their appropriate chirality (red or blue) and apparently randomly adopt one of the two chiral forms (red or blue) or a third, symmetrical (black) form (Theisen et al., 1994; Zheng et al., 1995; Krasnow et al., 1995; Tomlinson et al., 1997). Thus these mutations appear to prevent the ommatidia from reading the polarizing signals correctly.

We therefore asked whether we could determine which of the genes we were examining were involved in both the polarity read-out step and the preceding polarizing mechanism. Since Dsh was clearly involved in the read-out mechanism and was also usually required for Wg signaling (Siegfried et al., 1995; Krasnow et al., 1995; Tomlinson et al., 1997). Thus these mutations appear to prevent the ommatidia from reading the polarizing signals correctly.
1994), we asked whether Dsh was also involved in the earlier Wg-related step that was sensitive to arr, Arm and Sgg.

Analysis of Dishevelled

*dishevelled* null clones induce limited polarity reversals on the equatorial side

We examined a large number of *dsh* clones in the eye looking for non-autonomous effects. Since *arr* and *arm* clones had shown their greatest potency in the polar regions, we examined *dsh* clones in these positions and observed clear non-autonomous effects (Fig. 7A) with polarity inversion evident in the tissue lying equatorial to the clone. Clones elsewhere in the eye rarely showed non-autonomous effects, but when they did then the polarity inversion was on the equatorial side (Fig. 7B). Thus *dsh* clones induce polarity reversals on the equatorial side, which is consistent with a role for Dsh in regulating the activity of the inferred factor-X, but the weakness of the effect suggests that Dsh is functional only partially here.

One explanation for the weakness of the Dsh function detected here was that residual gene function persisted in the allele (*dsh*V26) that we used. This allele had previously been reported to contain a gene-internal deletion (Klingensmith et al., 1994). To further characterize the molecular lesion in this allele, we sequenced the breakpoints and established that 3' to the deletion the coding sequence is out of frame (see Materials and Methods).

Since Dsh functions in the read-out mechanism the chirality within the clones are random and the formation of ectopic equators as in *arr* or *arm* clones therefore could not be assessed. However, we were able to use the equatorial enhancer trap (Eq-1) as a marker to test for such equatorial quality in *dsh* clones.

*dsh* clones weakly express the Eq-1 marker

When *dsh*V26 clones were induced in a fly carrying the Eq-1 marker, ectopic expression of the equatorial marker was evident, but significantly reduced compared with *arr* (Fig. 5D). Thus both with the extent of the polarity inversions and the ectopic expression of the equatorial marker, *dsh* gene function appears to play a limited role in this initial polarizing mechanism.

Which proteins are used in the read-out step?

We expected the phenotype of mutations in the read-out mechanism to be similar to *fz* and *dsh*, that is the clones would contain ommatidia of random chirality. However, *sgg* clones do not differentiate as retinal tissue and therefore cannot be scored for chirality. With Arm, by contrast, we were able to make clones that contained ommatidia able to ‘read’ the polarizing signals as evident from their orderly arrangement into two domains of red and blue ommatidia (Fig. 6A). However, since these were effectively hypomorphic clones, then the residual gene function may have complemented the role of *arm* in the read-out step. Thus it was unclear to us whether Arm and/or Sgg operated in the read-out step.

Another way to probe the read-out mechanism is to use the *sevenless* enhancer element (Basler et al., 1991) by misexpressing proteins and assaying for chirality effects. The *sevenless* enhancer is active early in ommatidial development when the polarity read-out step occurs and drives transcripion at a high level. When wild-type copies of the frizzled (*fz*), *dishevelled* (*dsh*) or shaggy (*sgg*) genes are expressed in this way then the ommatidia adopt random chiralities resulting probably from a compromised read-out mechanism (Tomlinson et al., 1997). Thus from this previous analysis, data suggested that Sgg was involved with *fz* and *dsh* in the polarizing read-out mechanism. We now asked whether misexpression of Arm could similarly affect ommatidial chiralities.

Misexpression of Arm under *sevenless* enhancer control does not disturb ommatidial chirality

When Arm was expressed under *sevenless* transcriptional control, we saw no evidence for chiral changes, even when four copies of the transgene were present in the fly (Fig. 6C). We then tested activated Arm under *sevenless* control and again saw no evidence for chirality problems. The analysis with the activated Arm was complicated by the fact that the construct induced severely disrupted ommatidia, frequently devoid of photoreceptors and the retinal tissue appears to be made largely of pigment cells. However, when expressed in clones, at the interface with the wild type tissue genetically mutant photoreceptors form. These ommatidia were always of the appropriate chiral form, and we infer that Arm does not influence the chiral choice mechanism in a similar manner to *fz*, *dsh* and Sgg.

Our results suggested that Arm functioned in the polarizing mechanism but was not involved in the read-out step. We were unable to express *arr* with the *sevenless* assay and test for a role in the read-out step since the gene is not available. We then asked whether we were able to assay any role for *fz* in the polarizing mechanism. *fz* clones show non-autonomous effects but these are highly short-range and limited to the polar side of the clones (Zheng et al., 1996). The significance of this non-autonomy is unclear but, since it occurs on the polar side of clones, we inferred that it is not involved in the mechanism that regulates factor-X activity. Since the Eq-1 enhancer becomes ectopically expressed in *arr* clones, we therefore place this marker downstream of the primary organizing mechanism. To test whether *fz* is involved in the primary organizing mechanism, we examined whether the Eq-1 enhancer was affected in *fz* mutant eyes.

Placing Frizzled downstream of the polarizing signals

When the Eq-1 enhancer is crossed into a *fz* null fly, its expression pattern remained unchanged (Fig. 5C). If *fz* is involved in the polarizing step then we would have expected to see changes in the expression of this equatorial marker as we observe with *arr* and *arm*. Since we do not, we place the Eq-1 expression upstream of *fz* and downstream of the primary polarizing mechanism.

Does Wg normally function as the primary organizer of the Eq/Pl axis?

The experiments that we have described demonstrate the ability of Wg to polarize the Eq/Pl axis of the retina. Additionally we have demonstrated that elements of the Wg transduction mechanism appear to mediate this polarizing activity by regulating the activity of a secondary signal – factor-X.

We now asked whether Wg functions to organize the Eq/Pl axis during normal eye development or whether its ectopic effects mimic the activity of the bona fide organizing molecule.
wg\textsuperscript{+} is expressed in the tissues bordering the presumptive polar regions of the eyes disc ahead of the morphogenetic furrow, but not in the main body of the developing retina (Ma and Moses, 1995; Treisman and Rubin, 1995). So its expression domain makes it a likely candidate for an Eq/Pl-organizing molecule. We first removed wg gene function from the eye in clonal patches and, consistent with the absence of its transcription from this tissue, we observed only infrequent and minor patterning defects (data not shown). To look for a role of the polar expression of Wg, we removed wg gene function during larval life by shifting flies carrying the wg\textsuperscript{el} allele to the restrictive temperature. This experiment was not informative in defining a role for Wg in the Eq/Pl axis for two reasons. First, loss of wg gene function in this manner can cause major reorganizations of the head capsule tissue, leading to severe distortion of the retina and therefore preventing analysis of the Eq/Pl polarity. Second, loss of wg gene function induces ectopic morphogenetic waves to move in the Eq/Pl axis (Ma and Moses, 1995; Treisman and Rubin, 1995), obscuring any concomitant reversal in the Eq/Pl axis.

We then asked whether any of the three other identified fly Wnt proteins (Drosophila Wnt-2, Wnt-3, Wnt-4; Eisenberg et al., 1992; Russell et al., 1992; Graba et al., 1995) could also cause the polarity reversal seen with Wg. We induced clones in the eye of each of the three Wnts expressed under the control of the tubulin promoter. None of the three were able to phenocopy the effects of Wg expressed under these conditions. This suggests that Wg or another untested protein is responsible for organizing the Eq/Pl axis.

**DISCUSSION**

The results that we present in this paper make five major points.

1. The graded activity of Wg (or a closely related molecule) over the Eq/Pl axis functions as the primary organizing mechanism of polarity.

2. The graded activity of the primary organizing influence functions to set up the graded activity of a secondary molecule we refer to as factor-X.

3. This graded activity of factor-X likely represents the polarity information that is ‘read’ by the ommatidia.

4. The primary polarizing mechanism is mediated by Arm Sgg and Dsh and so appears as a Wg transduction, although Dsh appears to be only weakly used. The read-out of factor-X utilizes Fz, Dsh and Sgg, but apparently not Arm.

5. The polarizing mechanism for the Eq/Pl axis is separable from the establishment of A/P polarity and thus we propose that the *Drosophila* retina is an epithelium polarized in two orthogonal axes.

**Is Wg the primary organizer of the Eq/Pl axis?**

The misexpression of Wg in the developing eye has two important features. First, the associated polarity reversals occur exclusively on the polar side (rather than the equatorial side) and the retina is more sensitive to ectopic Wg at the equator than at the poles. From this we infer that the normal polarizing influence has a graded activity that is high at the poles and low at the equator and that the direction of the gradient supplies polarized information in the Eq/Pl axis (Fig. 8A–C).

The most likely molecule responsible for this graded activity is Wg itself. It is transcribed immediately adjacent to the developing retina in the polar regions (Ma and Moses, 1995; Treisman and Rubin, 1995) and, from this position, we envisage that Wg can diffuse from its site of secretion and achieve a graded distribution in the Eq/Pl axis of the retina (a similar proposal was previously made by Treisman and Rubin, 1995). Wg is known to influence cells many diameters away from its source of secretion (Zecca et al., 1996) and assuming that this signaling occurs early when the retina is small then it is possible for Wg to achieve a graded influence over the entire retina. However, we have been unable to demonstrate a requirement for wg in the Eq/Pl axis and the phenomena that we observe when we misexpress Wg may result from it mimicking the activity of another Wnt molecule. To investigate this we misexpressed the other identified fly Wnts in a manner similar to Wg and observed no polarity inversions.

Evidence from recent publications support a role for Wg in organizing the Eq/Pl axis. Reduction or ectopic expression of Wg can cause a reorganization of the eye disc as visualized with markers that are normally restricted in the Eq/Pl axis. Reifegerste et al. (1997) show that two equatorial markers (Eq-1 and PD) become expressed in polar regions of the eye disc when Wg signaling is reduced. Eq-1, the equatorial marker that we describe here, is ectopically activated in arr clones (Fig. 5) and this provides indirect evidence that Wg organizes the Eq/Pl axis. Heberlein et al. (1998) using a number of markers different from those described above show clear evidence of a role for Wg in directing differential gene expression in the Eq/Pl axis. Thus, although our analyses using ommatidial chirality as a meter of polarity have failed to confirm a role for Wg in organizing the Eq/Pl axis, the effects of ectopic Wg expression that we present here, combined with the results of Reifegerste et al. (1997) and Heberlein et al. (1998), suggest that Wg is the most likely candidate for the primary organizing molecule.

**The Wg-related signaling appears to controls a second secreted molecule with ability to polarize the Eq/Pl axis**

Clones of mutations in arr, arm and dsh to a variable extent induce polarity inversions on their equatorial side. Why they show different potencies will be discussed below, but the critical observation is that mutations in these recognized transducers of the Wg signal induce non-autonomous effects, consistent with them regulating the activity of a secondary signaling factor. We term this secondary signal as factor-X. Not only do arr, arm and dsh clones specifically affect the equatorial side, they are also more potent in achieving this at the pole rather than the equator. Thus we infer that factor-X activity is graded in the Eq/Pl axis but we do not have sufficient information to determine whether it is high at the equator and low at the poles, or vice-versa. Fig. 8D–F shows our interpretations of the effects of arr clones under these two different scenarios. If factor-X is positively regulated by arr then the activity distribution will be high at the pole and low at the equator. Conversely if arr negatively regulates, then factor-X will be high at the equator.

**The variable effects on factor-X activity of the transducers of the Wg-like signal**

arr mutant clones display the strongest non-autonomous effects on Eq/Pl polarity whereas arm and dsh clones are considerably
Figure 1:  

(A) Ectopic Wg  

(B) Ectopic Wg  

(C) Ectopic Wg  

(D) Ectopic Wg  

(E) Ectopic Wg  

(F) Ectopic Wg  

(G) Polarizing Step: Wg(?)  

Read-out Step: FAC-X  

Chirality: Fz Dsh Sgg Arm
Fig. 8. Models for the roles of Wg and Factor-X in organizing Eq/Pl polarity. The upper level (A-C) depicts the model for Wg function and the middle level (D-F) shows how the Wg-related signal may function to achieve a graded distribution of factor-X activity in the Eq/Pl axis. The lower level (G) depicts the two step cascade that organizes polarity. (A) Wg is expressed in positions flanking the poles and not in the eye itself (Ma and Moses, 1995; Treisman and Rubin, 1995). Polarizing effects from Wg would influence the retina ahead of the advancing wave front and establish a high/pole to low/equator distribution. (B) When Wg is ectopically expressed in the eye, a supernumerary high point of Wg signal is induced the local Wg distribution is disturbed causing polarity reversals on the polar side. Note that this leads to the formation of a pseudo-equator with the clone. (C) When Wg is ectopically expressed at the midline, the entire equatorial region is reorganized and polarity inversions occur in both halves of the eye. The midline is now represented by a pseudo-equator, flanked either side by the polarity reversals that induce ectopic equators at the junction with the wild-type tissue. (D-F) The upper row shows the distribution of factor-X activity if it is positively regulated by arr, and the lower row shows the situation for negative regulation. In either case, loss of arr function causes the clone in a cell autonomous way, to adopt the equatorial condition for factor-X. Diffusion then distributes factor-X activity in a graded manner and in both scenarios the polarity reversal will now be seen on the equatorial side of the clone and ectopic equators form at their centers.

less potent. We propose that these effects result from both differential usage of these molecules and from the strength of alleles used. We were unable to generate scorable null arm clones in the eye. This most likely resulted from cell-lethality problems associated with the structural role Arm plays in the cell. We therefore used a partial rescuing construct to supply low levels of wild-type Arm protein (Peifer et al., 1991). In this balanced situation, we presume that enough Arm protein is available to rescue the essential structural function but insufficient to fully regulate the activity of factor-X. Hence the clones of arr that we analyzed in the eye were far from null and accordingly we propose that this accounts for the weakness of their affect. Conversely, the dsh(V26) allele that we used is very strong and probably null (Klingensmith et al., 1994; this paper) and yet it showed significantly reduced non-autonomous effects compared to arr. We therefore suggest that this Wnt transduction mechanism is only partially Dsh-dependent and that another molecule supplies the function not provided by Dsh.

An alternative hypothesis is that the weak effects that we observe with the reduced Arm levels represent the full extent of Arm function in this pathway and that Arm and Dsh play similar roles mediating significantly less polarity information than arr.

The read-out of planar polarity and the molecular nature of factor-X

The Wg-related signal functions to produce graded activity of factor-X in the Eq/Pl axis of the developing retina and we propose that it is the direction of change in factor-X activity that is read by the emerging ommatidia. The sevenless enhancer assay allows us to probe for a protein’s function in the read-out step by overexpressing it at a critical stage of ommatidial development. By this method, we have implicated Fz, Dsh and Sgg in the read-out mechanism but not Arm.

The Wnt receptors are Frizzled class proteins and Fz itself has been shown to bind Wnts (Bhanot et al., 1997). This then raises the possibility that factor-X is a Wnt and, if this is the case, then its second messenger system appears to be arm independent. Further evidence for the Fz transduction pathway being not of the standard Wnt type comes from the work of Strutt et al. (1997) that suggests that Fz signaling is mediated by the GTPase RhoA.

Multiple roles of the Wg signaling mechanism in eye development

The work of Ma and Moses, (1995) and Treisman and Rubin, (1995) assigned to Wg a critical role in regulating where ommatidial differentiation was initiated in the disc. The dorsal and ventral Wg expression (the polar expression in the tissue bordering the eye) functions to suppress precocious ommatidial differentiation in these positions and thereby prevent the formation of ectopic waves of eye patterning running in the dorsoventral axis. Furthermore, Treisman and Rubin (1995) suggested that Wg also acts to suppress premature initiation of the ommatidial differentiation program in the main body of the retina ahead of the advancing wave front. That is, a long-range influence of Wg at the poles would prevent the retinal tissue from differentiating prior to the arrival of the inductive morphogenetic wave. Evidence for this came from ectopic expression of Wg in the eye and the effect of sgg clones both of which suppressed ommatidial differentiation.

We too have observed these effects and offer further evidence in favor of this model. First, the expression of activated Arm mimics ectopic Wg in that it has potent abilities to prevent ommatidial differentiation, whether expressed constitutively or under sevenless control. We note however that there is significant rescuing non-autonomous effect of the wild-type tissue surrounding the clones. Second, clones of arr and arm (transducers of the Wg signal) frequently appeared to trigger the precocious differentiation of the ommatidia causing similar effects to loss of ptc or pka or premature expression of Hedgehog (Heberlein et al., 1995; Ma and Moses, 1995; Strutt et al., 1995; Wehrli and Tomlinson, 1995). Thus there is mounting evidence that the Hedgehog signal from the advancing wave front antagonizes a Wg or Wg-like repression to elicit timely differentiation of the ommatidia.

Cruciform planar polarity in the Drosophila eye

The planar polarity in the Drosophila retina is evident as the chirality and orientation of the ommatidia. A two-dimensional organization is required to generate the ommatidial array, whereas a single piece of directional information can orient hairs or bristles in standard planar polarity. We proposed earlier (Wehrli and Tomlinson, 1995) that ommatidial chirality was directed by two independent and orthogonal signaling mechanisms, one lying in the A/P axis and the other Eq/Pl (Fig. 1). The manipulation of the morphogenetic wave that sweeps anteriorly across the developing retina frequently caused inversions in the A/P axis without affecting the Eq/Pl axis. However, other data suggested that sometimes both the A/P and Eq/Pl axes could be affected by the reversal of the morphogenetic wave. Why different effects can be observed is not clear and, in order to resolve this issue, we began an investigation of the Eq/Pl axis. The manipulations of the Eq/Pl polarity that we describe here argue that this axis is organized independently of the A/P axis and we propose that two signaling systems impose separate planar polarities on the retina. Reifegerste et al. (1997) infer the presence of a second
polarizing axis in the retina that is independent of the A/P polarity and Heberlein et al. (1998) conclude that there is Eq/PI information distinct from and ahead of the advancing wave front. Since the resulting axes lie at right angles to each other, we describe this phenomenon as ‘cruciform planar polarity’. We envisage that a group of cells destined to form an ommatidium respond to each axis of information and the shape of the ommatidium that forms depends upon the combined action of the two signals that the cells experience (Fig. 1D).

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